Studies on Enzyme Action. VII.—*The Synthetic Action of Acids contrasted with that of Enzymes. Synthesis of Maltose and Isomaltose.*

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The belief has grown up of late years that the enzymes which are capable of inducing the hydrolysis of disaccharides or bioses act reversibly; as yet, however, but little has been done to define the theory of the process and no understanding has been arrived at as to the limitations to which such changes are subject. The same is true of the action of acids, which also act reversibly under certain conditions.

Croft Hill,* whose observations gave rise to the conception of reversible enzyme action, at first thought that maltose alone was produced by the action of the enzyme maltase on glucose. Emmerling,† who repeated Croft Hill's experiments, came to the conclusion that the product was isomaltose, the biose which E. Fischer‡ obtained by subjecting glucose to the action of concentrated chlorhydric acid. In a later communication,§ while still claiming that maltose is formed in small quantity, Croft Hill has admitted that the chief product is an isomeride of maltose; but he regards this as different from isomaltose and therefore terms it *revertose.*

By subjecting a mixture of galactose and glucose such as is obtained by hydrolysing milk sugar with lactase to the action of this enzyme, a disaccharide, isolactose,‖ is formed, which is undoubtedly isomeric with milk

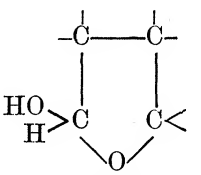
* 'Chem. Soc. Trans.,' 1898, p. 634; 'Ber.,' 1901, vol. 34, p. 1380.
† 'Ber.,' 1901, vol. 34, pp. 600 and 2206.
‡ 'Ber.,' 1890, vol. 23, p. 3687; 1895, vol. 28, p. 3024.
§ 'Chem. Soc. Trans.,' 1903, p. 578.
‖ E. Fischer and E. F. Armstrong, 'Ber.,' 1902, vol. 35, p. 3151.

sugar, as the synthetic product is completely fermented by bottom yeast, a yeast having no action on milk sugar; moreover, as the solution has no reducing action after the removal of this product by fermentation, the presence of milk sugar is precluded.

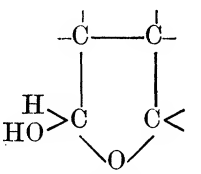
This observation is scarcely compatible with the statement made by Croft Hill in his latest communication that, whilst a large number of isomeric bioses are conceivably possible, "one would expect that with any particular enzyme or group of enzymes only those would be formed which are capable of being hydrolysed back to glucose by the same enzyme:" the very opposite would appear to be the case, as will be shown later on.

If the argument made use of in previous communications be correct, the action of acids and of enzymes, both as hydrolytic and as condensing agents, is similar, except that, and in so far as, differences arise owing to the non-selective activity of the former and the strictly selective activity exercised by the latter.

The key to the interpretation of the changes which attend condensation must be looked for in the behaviour of glucose itself in solution. It is now abundantly proved that glucose can exist in two stereoisomeric forms, differing only in the position which the hydroxyl group occupies relatively to the oxygen atom in the ring, viz.:—



α-Glucose.*



β-Glucose.

The term glucose, in fact, has a double connotation and these two substances must usually be thought of under the single name. As crystallised from alcohol, it consists almost entirely of the α-form; but this changes over into the β-form if maintained during several days at about 105°.† If either form be dissolved in water, change takes place of the one into the other: ultimately, the two forms exist in solution in equilibrium, in proportions which depend on the conditions, the β-compound predominating.‡

* The two positions are labelled arbitrarily.

† *Cf.* Tanret, 'Bull. Soc. Chim.,' 1905, vol. 33, p. 337.

‡ Tanret, who was the first to recognise that glucose existed in several forms, has been led by the results recorded in No. 1 of this series to reconsider the conclusion he originally came to that three isomerides are obtainable. He now agrees that there are but two, corresponding to the two α- and β-methylglucosides, and that his supposed third modification was an equilibrated mixture of these two forms. Calculating from the rotatory power ($[\alpha]_D = +110°$) of the pure α- and $[\alpha]_D = +19°$ of the pure β-form, he

Change takes place in a similar manner in other media. If the glucose be dissolved in methylic alcohol containing hydrogen chloride, it undergoes etherification, each of the two glucoses being converted into the corresponding methylglucoside:* only one of these (the α-compound) is hydrolysed by maltase; the other (the β-compound) is hydrolysed by emulsin. The behaviour of these two glucosides is typical of that of glucosides generally, which are divisible into two groups, the α and the β, according as they are hydrolysed either by maltase or by emulsin. It is noteworthy that, apart from the sugars proper, in all cases in which the test can be applied, the natural glucosides have been found to belong to the β-group.

The process by which a monose is converted into a biose must be regarded as precisely similar to that by which α-glucose and β-glucose are converted into the two methylglucosides: the behaviour of maltose, in fact, is such as to characterise it unquestionably as glucose-α-glucoside; isomaltose is presumably the stereoisomeric glucose-β-glucoside.

When glucose undergoes condensation "uncontrolled," it should give rise to both maltose and isomaltose, the proportions of which ultimately present in equilibrium would depend on their relative stability under the conditions operative at the time. But, inasmuch as hydrolysis under the influence of enzymes is an absolutely selective process, being so controlled that it takes place in one direction only, it might be supposed that synthesis under their influence would also be a controlled operation and that the tendency of the enzyme would be to reproduce the biose which it hydrolyses: apparently this point of view was present in Croft Hill's mind and led him to suppose, at first, that maltose was the actual product; as a matter of fact, it is uncertain at present whether maltose is produced at all: it is certainly not the sole nor even the predominant product.

The formation under the influence of the enzyme of a single biose, *isomeric with that which it hydrolyses*, could be accounted for on the assumption that both are produced initially but that the one again undergoes hydrolysis as soon as it is formed, so that it all but disappears. If, however, it were shown that only the stereoisomeride of the biose hydrolysed is produced initially, it would be necessary to regard the synthetic activity of the enzyme as opposed to its hydrolytic activity. An explanation which in a measure unites both

infers that the proportion in which these are present in equilibrium is $\alpha = 37$ per cent., $\beta = 63$ per cent. in a 10-per-cent. solution, and $\alpha = 40$, $\beta = 60$ in a concentrated solution.

Lowry ('Trans. Chem. Soc.,' 1904, p. 1551), who bases his conclusions on determinations of solubility, takes the view that a solution of glucose contains a considerable proportion of glucose aldehydrol in addition to α- and β-glucose; but his argument cannot be regarded as a convincing one ('Comp. Jungius. Zeit. Phys. Chem.,' 1905, p. 103).

* E. F. Armstrong and S. L. Courtauld, 'Proc. Physiol. Soc.,' July, 1905.

points of view is that the enzyme acts throughout by "protecting" one or the other position, according as it belongs either to the α- or to the β-class of hydrolysts, thereby practically preventing condensation from taking place in more than one direction; in other words, assuming that it be the function of the enzyme to bring water into the circuit of change at the precise spot where it is required to effect the hydrolysis of a biose, it might serve, when acting on the products of hydrolysis, to maintain water in such a position as to hinder the condensation from occurring in the direction which would involve the reversal of the operation of hydrolysis; condensation would then be confined to the alternative position and would give rise to the biose which is the correlative of that hydrolysed by the enzyme.

There can be no doubt that the enzyme has a specific influence in promoting the formation of the biose which it cannot hydrolyse, as no action takes place in its absence or when the solution is heated sufficiently to destroy it. To understand the character of this influence, it should be remembered that α- and β-methylglucoside have both been shown to be capable of entering into close association with maltase and with emulsin; presumably, therefore, the two forms of glucose present in solution both combine with the enzyme. Inasmuch as the enzymes are capable of acting as hydrolytic agents, they must, like acids, be capable also of acting as dehydrating agents. Probably the two forms of glucose give rise to different results, because, while hydrolytic action prevails in the one system, in the other the dehydrating effect is alone exercised, the association of the α-enzyme with α-glucose being of such a nature that water is continually present and can be made use of at the centre where the condensation should take place, the dehydrating effect being therefore almost entirely in abeyance; whilst in the enzyme-β-glucose system the configuration of the enzyme relatively to the β-glucose is such as to render hydrolysis impossible and consequently the dehydrating effect prevails. In both cases opportunity conditions action: the different actions are begotten of different opportunities.

It is noteworthy that, under natural conditions, apparently only one product is formed, there being no evidence, for example, that isomaltose is ever present in the plant. If further investigation should render this conclusion absolute, it will follow that the control exercised under natural conditions is not merely that which the separated enzyme exercises. But attention must not be confined to the enzyme, as the argument used above tends to show that, even under the influence of maltase, maltose alone might be produced, if the conditions prevailing in the plant be such as to give rise only to α-glucose, provided that the maltose were immediately withdrawn from the sphere of action, for example, by diffusion and fixation as starch;

and that such may be the case is by no means improbable, as it is conceivable that if glucose were formed against a maltase template,* it would be present initially only in the α-form.

It will be obvious from these statements that it is all-important to determine the nature of the product, both when condensation is effected by ordinary chemical means and also when effected by means of an enzyme.

1. Proof is given in the present communication that when the condensation is effected under laboratory conditions the action takes place in the manner indicated above; in other words, the two products required by theory are both formed.

2. Evidence is adduced to show that isomaltose is the β-glucoside correlative with the α-glucoside maltose.

3. Experiments are described bearing on the formation of isomaltose by the agency of the α-enzyme maltase and of its correlative maltose by the agency of the β-enzyme emulsin which leave little doubt that the two bioses are producible from glucose.

4. And whilst it is left undecided whether maltase can give rise to maltose, evidence is cited which at least renders it probable that emulsin does not give rise to isomaltose.

Synthesis of Maltose and Isomaltose by means of Chlorhydric Acid.

To effect the condensation of glucose, E. Fischer used ordinary chlorhydric acid. The precipitate obtained on mixing the liquid with a large quantity of alcohol and ether was dissolved in water and all fermentable matter was removed from the neutralised solution by means of brewers' yeast. Such a method of purification would have destroyed any maltose which had been formed. Fischer does not appear, however, to have contemplated the formation of this sugar.

In my experiments, a stronger acid was used and this was afterwards removed by means of lead carbonate. Appropriate yeasts were used to destroy one or the other carbohydrate.

One hundred grammes of glucose having been dissolved in 300 c.c. of concentrated chlorhydric acid, the mixture was cooled to 0° and hydrogen chloride gas was passed in until the colour commenced to darken. The liquid was kept below +10° C. during about 40 hours, when the temperature was allowed to rise to 15°. After neutralising the acid by stirring the liquid with lead carbonate, the filtrate and washings were shaken with silver carbonate to remove the dissolved lead chloride.

Finally an almost colourless neutral solution was obtained. Judging from

* 'Roy. Soc. Proc.,' 1904, vol. 73, p. 538.

its behaviour with phenylhydrazine, this contained a biose, together with much glucose. The manner in which isomaltose and maltose were detected will be apparent from the following descriptions:—

Proof of the Presence of Isomaltose.—To remove the unchanged glucose, about 20 c.c. of boiled yeast-water were added to the liquid, which was then sterilised and inoculated with a quick-acting pure yeast, *S. intermedians*, Hansen. After fermentation had gone on during 10 days at 25°, the solution was filtered, mixed with a little calcium carbonate to neutralise any acid which had been formed during the fermentation, boiled to expel alcohol and then reinoculated with yeast under sterile conditions. These operations were usually repeated a second time, experience having shown such repetition to be the only way in which the last trace of fermentable sugar can be removed.

The solution finally obtained, when clarified with charcoal, was almost colourless; the total volume was 150 c.c.; its rotatory power in a 1-decimetre tube was $\alpha_D = 4°{\cdot}20$.

When a mixture of about 15—20 c.c. of the solution with 2—3 c.c. of almost colourless phenylhydrazine, dissolved in 3 c.c. of 50 per cent. acetic acid, was heated in a flask in a boiling water bath during 1—1½ hours, no separation of osazone took place from the hot liquid, which was an indication that no glucosazone was formed. On cooling the liquid, a light yellow flocculent osazone separated out slowly; this was filtered off, well washed with cold water and redissolved in boiling water, in which it was entirely and easily soluble. The osazone only crystallised out when the solution was nearly cold, differing in this respect from maltosazone, which crystallises out while the solution is still hot. After a second crystallisation from water, the osazone was crystallised from dilute alcohol; it then formed a light brownish yellow crystalline powder, which decomposed when heated at about 120°. To complete the purification, it was next crystallised from wet ethylic acetate. The slightly yellow micro-crystalline powder thus obtained was very soluble in boiling water; on heating, it melted at about 156°, forming a brown liquid, which decomposed at 198°, behaving in every way exactly in the manner described by E. Fischer.

It was to be supposed that if it were a derivative of glucose, it would be hydrolysed by emulsin: 30 c.c. of the solution were therefore mixed with a small quantity of active emulsin and a few drops of toluene; the liquid was then set aside in a closed flask during several days at 38°, along with a similar quantity of the solution to which no enzyme had been added. To test whether glucose had been formed, the filtered solutions were heated side by side with phenylhydrazine. In the one case, separation of an insoluble

glucosazone was observed after 20 minutes' heating; in the control experiment no separation took place but a soluble osazone was formed.

Proof of the Presence of Maltose.—In order to destroy the glucose, the liquid was fermented with *S. Marxianus*, a yeast which does not contain maltase and therefore is without action on maltose. After removal of the fermentable matter, the solution, 200 c.c. in volume, containing the product from 50 grammes of maltose, had the rotatory power $\alpha_D = +7°{\cdot}35$, which was about double that observed in a similar solution containing isomaltose alone. The osazone formed on heating with phenylhydrazine was entirely soluble in boiling water, but distinctly less soluble than the isomaltosazone; it began to separate from the liquid while this was still hot and the osazone could be fractionated into more and less soluble portions.

Separate portions of the solution were digested with emulsin and maltase; in both cases glucoses were formed. When subjected to the joint action of emulsin and a yeast containing maltase (*S. intermedians*), the solid matter in solution disappeared almost entirely in a single fermentation.

The isomaltose is apparently present in larger quantity than maltose: it is proposed to determine their relative proportions by removing the isomaltose from the mixture by the joint action of emulsin and *S. Marxianus*.

The Production of Isomaltose by means of Maltase.

The maltase extract used was always prepared by grinding 5 grammes of air-dried top yeast (from a London brewery) with 100 cm. of water and then digesting the mixture at 25° during two to three hours. Fifty grammes of glucose were dissolved in about 75 c.c. of the filtered extract, and some toluene was added to maintain the liquid sterile; the solution was kept in a stoppered bottle at 25° during two to three months or even longer; it darkened somewhat but remained perfectly clear and sweet. To remove glucose and maltose, the solution diluted with an equal volume of water was boiled with charcoal and filtered; yeast water was then added and after the liquid had been sterilised it was fermented with *S. intermedians*.

To insure complete removal of the glucose, it was necessary to repeat the fermentation at least twice. Ultimately, a clear solution was obtained which not only had a strong reducing action on Fehling's solution but also a high positive rotatory power. When treated with phenylhydrazine in the manner described, it gave an osazone in every way identical with isomaltosazone; in fact, when the experiments were carried out side by side with the products obtained by means of acid and by means of the enzyme, no difference could be detected. The product also behaved in the manner to be expected towards emulsin, being converted into glucose.

The Production of Maltose by means of Emulsin.

A solution of 50 grammes glucose in 75 c.c. water was mixed with 1 gramme of emulsin. The liquid was kept at 25° during about two months: then filtered, diluted and freed from glucose by continued fermentation with *S. Marxianus* in the manner described.

Ultimately, a solution was obtained which contained a disaccharide yielding a phenylosazone soluble in boiling water but separating quickly on cooling; the product crystallised in plates, which, when recrystallised from wet ethylic acetate, melted and decomposed at 200°, which is but very few degrees below the melting point of pure maltosazone.

The matter in solution underwent almost complete fermentation when inocculated with a yeast containing maltase (*S. intermedians*); only traces of reducing sugar remained, showing that little, if any, isomaltose could have been formed.

These observations leave practically no doubt that the substance present was maltose. The yield under the conditions hitherto observed has been very small.

The experiments will be continued with the object of obtaining both maltose and isomaltose in a separate crystalline state.